Cellular fatty acid composition of symbiotic cyanobacteria isolated from the aquatic fern *Azolla*

The cellular fatty acid composition of 10 isolates of symbiotic cyanobacteria from 7 different species of the aquatic fern *Azolla* was investigated. Sixteen major components accounted for 88·31% of total fatty acids: the saturated 14:0, 16:0 and 18:0 carbon chains; the unsaturated straight-chained 12:1, 14:1 *cis*-7, 16:1 *cis*-7, 16:1 *cis*-9, 16:1 *cis*-11, 18:2 *cis*-9, 18:3 *cis*-9, 18:1 *cis*- and *trans*-9, and 20:4 *cis*-5; and the branch-chained *iso*-16:0. Also included was an unsaturated 16-carbon (equivalent carbon chain length of 15·5), with unsaturation sites undetermined. The most abundant component was the 16:0 (mean of 38·10% of the total). Thirty-six minor fatty acids, comprising 10·30% of the total, were detected and identified. These included hydroxy-substituted fatty acids (1·10%), branched chains in addition to the *iso*-16:0 (1·96% of a class total of 2·96%) and cyclopropane fatty acids (0·89%). A comparison of the fatty acid profile of *Azolla* cyanobionts with those previously published for free-living cyanobacteria of the genera *Anabaena* and *Nostoc* indicated that there were at least 19 individual fatty acids, class totals or ratios that were statistically different and could be used as differentiating factors. Nine of the 19 factors were characteristically unique to *Azolla* cyanobionts and different from both *Anabaena* and *Nostoc*. Five were different from only *Anabaena*, and five from only *Nostoc*. Based on one taxonomic interpretation of fatty acid analysis, the *Azolla* cyanobionts appeared to be equally distinct from *Anabaena* and from *Nostoc*.

Introduction

Azolla Lamarck, an aquatic fern widely distributed in tropical and subtropical regions is host to symbiotic, nitrogen-fixing cyanobacteria (Lumpkin & Plucknett, 1982). Strasburger (1873) first described the *Azolla* cyanobiont, designating it *Nostoc*, and later renaming it *Anabaena azollae* (Strasburger, 1884). Its correct taxonomic classification has remained controversial.

The genera *Anabaena* and *Nostoc* have traditionally been differentiated by the morphology of a gelatinous sheath surrounding the trichomes, and by colony formation (Kirchner, 1900; Geitler, 1932). Rippka *et al.* (1979) suggested that the developmental life cycle should be the principal criterion in differentiating *Nostoc* from *Anabaena*. Reproduction in *Anabaena* is restricted to random fragmentation of trichomes or to germination of akinetes producing filaments of dimensions equal to those of parent cells. All trichomes in *Anabaena* are capable of gliding motility. Hormogonia, motile filaments composed entirely of vegetative cells smaller than the parental cells and with gas vacuoles in some species, can develop from akinetes in the life cycle of *Nostoc* (Rippka *et al.*, 1979; Castenholz, 1989; Meeks, 1990).

Cyanobacteria in the apex of *Azolla* fronds develop hormogonia as the main form of colonizing trichomes (Vashishta, 1975; Hill, 1975; Caudales *et al.*, 1990). Hormogonia are produced in older leaf cavities by akinete germination or by differentiation of vegetative cells adjacent to single or double heterocysts (Caudales *et al.*, 1990). Accordingly, based on these observations, on the criteria of Rippka *et al.* (1979) and Rippka (1988), and on the opinion of Peters & Meeks (1989) and Meeks *et al.* (1988), the *Azolla* cyanobiont is more closely related to the genus *Nostoc*.

Caudales & Wells (1992) reported that fatty acid composition was an effective taxonomic tool for differentiation between free-living *Anabaena* and *Nostoc*. The most useful parameters were the percentages of 16:0, of

saturated even-carbon straight-chain fatty acids (class A), and the ratio of unsaturated fatty acids (class C) to class A.

In this report we analyse the fatty acid composition of *Azolla* cyanobionts, and its relationship to that of *Anabaena* and *Nostoc*.

Methods

Media and growth conditions. Ten *Azolla* accessions were grown in defined mineral medium without nitrogen, modified from Van Hove *et al.* (1983) consisting of 54·4 mg KH_2PO_4 l^{-1}, 294 mg $CaCl_2.2H_2O$ l^{-1}, 192 mg $MgSO_4.7H_2O$ l^{-1}, 150 mg KCl l^{-1}, 12 mg NaCl l^{-1}, 12 mg Fe-EDTA l^{-1} and 1 ml trace metal mix A5 l^{-1} (Rippka *et al.*, 1979) plus 43·87 mg $CoCl_2.6H_2O$ l^{-1}. Photon flux density was 100 μmol m^{-2} s^{-1} from cool-white fluorescent and incandescent light, using a 14/10 h light/dark cycle. Temperature was 26 ± 1 °C during the light and 16 ± 1 °C during the dark periods. Names of the species, strains, geographical origins, and sources of accessions are listed in Table 1. Cyanobionts were harvested from 14–18 d old plants of 7 different *Azolla* species. Approximately 50 g of plants were washed in 0·2% sodium hypochlorite + 0·01% Triton for 20 min, rinsed ten times with distilled water, and macerated in phosphate-free $BG11_0$ medium (Rippka *et al.*, 1979) with a blender. Cyanobacterial cells were separated from plant debris by dilution of the macerate in medium, filtration through 10 layers of cheesecloth, and by differential centrifugation. Pellets were resuspended in $BG11_0$ medium containing 2% (w/v) each of pectinase and cellulase (Sigma) buffered at pH 6·0 with 10 mM-MES, and incubated for 2 h at 30 °C. Pellets were then washed twice in medium, centrifuged at 500 *g* (10 min), and fractionated by density gradient centrifugation in 10–90% (w/v) Percoll at 2500 *g* (20 min). Prior to fatty acid analysis, cyanobacterial preparations were removed, examined microscopically at 500× for plant debris using a Zeiss Universal microscope (Zeiss Optics, New York) and again washed twice in medium. If debris was present (over 10–20 particles per field), Percoll centrifugation and washing was repeated.

As a control sample, a cyanobiont-free isolate of *Azolla mexicana*, obtained from Dr E. Braun-Howland (Rensselaer Polytech. Institute, Troy, NY, USA) was macerated, (wet) weighed, diluted logarithmically, and each dilution examined microscopically. Dilutions were selected which corresponded closest to contamination levels found in some cyanobiont preparations (about 10–50 particles per microscopic field). These dilutions were then analysed for fatty acids to determine their contribution, if any, to cyanobiont profiles. Similarly, several types of bacteria from *Azolla* cavities, present as contaminants in some cyanobiont preparations, were cloned to single colonies, diluted to levels found in the preparations (less than 20 cells per field), and then analysed for fatty acids.

Fatty acid analysis. Approximately 400–500 mg (wet wt) of cyanobacterial cells were saponified and esterified by mixing in 1 ml 1·2 M-NaOH in 50% (v/v) aqueous methanol, heating for 30 min in a boiling water bath, then adding 0·5 ml 6 M-HCl and 1 ml 12% (w/v) BCl_3 in methanol, and heating for 5 min at 85 °C. Methylated acids were then extracted with 1 ml hexane-diethylether (1:1), washed with 3 ml 0·3 M-NaOH, and concentrated under a stream of purified, filtered N_2 to approximately 20–40 μl. A 2 μl sample was injected into a Varian 3700 Gas Chromatograph with a flame ionization detector and a 15 m × 0·25 mm capillary glass column coated with SPB-1 (Supelco) as a non-polar stationary phase. Solvent blanks were checked periodically for impurities. Operating conditions were: helium carrier gas flow of

Table 1. *Species and origin of Azolla host plants, and strain designations of their cyanobionts used in this study*

Species and strain	Original strain designation	Geographical origin	Source*
A. caroliniana			
C1	C1	Virginia, USA	G. Peters
C8	–	Río Chavón, Dominican Republic	Original isolate
A. rubra			
R3	204RU	Nouville, New Zealand	C. Van Hove
A. mexicana			
X1	X1	California, USA (BR-S-CF)	G. Peters
A. microphylla			
M2	M2	Galápagos, Ecuador (TL-GI-CF)	G. Peters
A. filiculoides			
F3	F3	Hawaii, USA (BR-M-CF)	G. Peters
F4	FLA	Hillboug, Tampa, Florida, USA	W. Zimmerman
A. nilotica			
N1	82NI/N1 /5001	Kosti, Sudan	Van Hove/Peters/ Zimmerman
A. pinnata var. *pinnata*			
P1	P1	Malaysia (BR-M-CF)	G. Peters
A. pinnata var. *imbricata*			
13	JNPI1	Chisato Mie, Japan	W. Zimmerman

* Sources: Dr Gerald Peters, Virginia Commonwealth University, Richmond, Virginia, USA; Dr Charles Van Hove, Universite Catholique de Louvain at Louvain-la-Neuve, Belgium; Dr William Zimmerman, University of Michigan, Dearborn, MI, USA.

30 ml min^{-1}; injector temperature, 230 °C; initial column temperature, 130 °C; final temperature, 230 °C; temperature program rate, 4 °C min^{-1}. These conditions yielded chromatograms containing at least 40 detected components (peaks) between 8 and 20 carbons in length. Each sample was analysed once, except for *A. rubra* which was grown, extracted and analysed on three separate occasions in order to determine experimental variability.

Fatty acids were identified by co-chromatography with reference standards that were available. Major fatty acids, defined as those constituting at least 0·9% of the total, were confirmed by mass spectrometer (Finnegan 8230 HR) and by chemical tests (Caudales & Wells, 1992). Other eluted peaks, comprising the minor fatty acids (0·03 to 0·89% of the total) and trace components (less than 0·03%) were confirmed chemically. Chemical tests included those for unsaturated, hydroxy-substituted and cyclopropane fatty acids (Moss, 1979). Reference standards for 21 bacterial fatty acids were obtained from Supelco and 12 branch-chain fatty acids from Applied Science. Equivalent carbon chain length (ECL) was calculated for each peak, and provided further confirmation of identity by reference to ECLs from published reports (Gillan & Hogg, 1984; Caudales & Wells, 1992). Unsaturated sites and their isomeric positions were directly determined by mass spectroscopy only in the case of major fatty acids.

Statistical analysis. Eluted fractions from gas chromatography were integrated and quantified as percentages of total peak areas with a Model 4270 Chromatography Data System (Varian Associates). Data were analysed by an Apple III computer running Omnis 3 Database Manager (Blyth Software). Fatty acids were categorized by chemical class (Asselineau, 1962) and subtotals calculated. Variations from the mean were expressed as standard sample deviations and comparisons among means were by the method of Tukey as modified by Snedecor (1966).

Results

The cellular fatty acid composition of 10 isolates of *Azolla* cyanobionts included 16 major components accounting for 88·31% of total fatty acids: the saturated

Table 2. *Mean percentage (of the total) of cellular fatty acids of ten strains of cyanobionts from Azolla spp.*

Fatty acids		Mean percentage of total*	
Chemical Class	ECL†	Percentage	Range‡
A. Saturated, even-carbon straight chains:			
8:0	8·0	0·04	0–0·06
10:0	10·0	0·03	0–0·05
12:0	12·0	0·25	0·12–0·41
14:0	14·0	1·10	0·66–1·38
16:0	16·0	38·10	35·17–42·16
18:0	18·0	2·33	1·20–3·55
20:0	20·0	0·19	0·10–0·29
Class A total		42·04	39·81–45·18
B. Saturated, odd-carbon straight chains:			
9:0	9·0	0·04	0–0·10
11:0	11·0	0·01	0–0·05
13:0	13·0	0·01	0–0·04
15:0	15·0	0·48	0·24–0·87
17:0	17·0	0·28	0·14–0·36
19:0	19·0	0·09	0·09–0·20
Class B total		0·91	0·59–1·43
C. Unsaturated, straight-chain acids:			
12:1	11·8	0·93	0·10–2·03
13:1	12·8	0·03	0–0·11
14:1 *cis*-7	13·8	0·12	0·01–0·32
14:1 *cis*-9	13·9	2·35	1·11–3·55
16:4	15·2	0·24	0–0·86
16:?§	15·5	1·36	0·54–1·87
16:4 *cis*-4	15·55	0·76	0·22–2·12
16:3 *cis*-6	15·6	2·51	0·97–4·31
16:1 *cis*-7	15·75	0·92	0–2·96
16:1 *cis*-9	15·8	4·45	3·31–5·34
16:1 *trans*-9	15·85	0·01	0–0·10
16:1 *cis*-11	15·9	1·96	0·44–2·54
16:1 *trans*-3	15·95	0·64	0·24–1·48
18:3 *cis*-6	17·2	0·55	0·27–1·02
18:4 *cis*-6	17·5	0·19	0·09–0·32
18:2 *cis*-9	17·7	8·36	5·75–14·00
18:3 *cis*-9	17·75	16·50	8·14–25·79
18:1 *cis*-9	17·8	3·81	2·95–5·18
18:1 *trans*-9	17·85	2·08	1·07–2·77
20:4 *cis*-5	19·2	0·95	0·40–2·09
20:4 *cis*-8	19·4	0·25	0–0·45
20:2 *cis*-11	19·6	0·36	0·10–0·55
Class C total		49·33	44·47–55·37
D. Hydroxy-substituted:			
20H-10:0	11·15	0·06	0–0·19
30H-10:0	11·4	0·17	0·04–0·39
iso 30H-11:0	12·1	0·02	0–0·13
20H-12:0	13·2	0·07	0–0·22
30H-12:0	13·5	0·04	0–0·14
iso 30H-13:0	14·1	0·15	0·11–0·29
iso 30H-15:0	16·1	0·04	0–0·18
iso 30H-17:0	18·1	0·22	0·01–1·23
30H-17:0	18·4	0·33	0·14–0·40
Class D total		1·10	0·53–2·31
E. Branch-chain acids:			
iso-13:0	12·6	0·09	0·01–0·15
iso-14:0	13·6	0·07	0–0·20
iso-15:0	14·6	0·06	0–0·18
anteiso-15:0	14·7	0·08	0–0·25
iso-16:0	15·6	1·96	0·41–1·96
iso-17:0	16·6	0·33	0·17–0·46
anteiso-17:0	16·7	0·19	0·10–0·25
iso-19:0	18·6	0·06	0–0·17
anteiso-19:0	18·7	0·12	0–0·30
Class E total		2·96	1·19–5·44
F. Cyclopropane acids:			
cyclo-17:0	16·9	0·17	0–0·55
cyclo-19:0	18·9	0·72	0–1·30
Class F total		0·89	0·07–1·38
G. Unsaturated branch-chained fatty acids:			
iso-15:1	14·4	0·05	0–0·15
iso-17:1	16·4	0·25	0·12–0·40
iso-18:1	17·4	0·36	0·10–0·94
Class G total		0·66	0·25–1·42
Unidentified components		2·11	0·88–3·12

* Mean percentages of total fatty acids.

† ECL = equivalent (carbon) chain length.

‡ Highest and lowest value for each fatty acid in the 10 strains analysed.

§ Component is unsaturated (determined chemically), but Site (or sites) undetermined.

Table 3. *Fatty acid parameters of Azolla cyanobionts significantly different from those of free-living Anabaena and Nostoc cyanobacteria*

Fatty acid or class total†	*Azolla* cyanobionts	Free-living cyanobacteria*	
		Anabaena	*Nostoc*
Class A total	42·04 ± 1·77 a	32·11 ± 2·45 b	25·02 ± 2·15 c
14:0	1·10 ± 0·26 a	0·44 ± 0·15 b	0·28 ± 0·14 b
16:0‡	38·10 ± 1·90 a	30·55 ± 2·25 b	23·23 ± 2·17 c
18:0	2·33 ± 0·74 a	0·77 ± 0·39 b	1·27 ± 0·59 ab
Class C total	49·32 ± 3·87 a	59·21 ± 4·99 ab	66·42 ± 4·22 b
14:1 *cis*-7‡	0·12 ± 0·14 a	2·50 ± 1·94 b	0·11 ± 0·21 a
16:1 *cis*-9‡	4·45 ± 0·62 a	6·53 ± 4·72 a	18·76 ± 4·72 b
16:1 *trans*-9	0·01 ± 0·03 a	4·02 ± 2·71 b	1·35 ± 0·96 a
16:1 *trans*-3	0·64 ± 0·35 a	0 b	0 b
18:2 *cis*-9	8·36 ± 2·37 a	10·16 ± 2·87 ab	12·44 ± 3·14 b
18:1 *trans*-9	2·08 ± 0·61 a	0·92 ± 0·51 b	1·94 ± 0·89 a
20:4 *cis*-5	0·95 ± 0·52 a	0·06 ± 0·11 b	0 b
Class E total	2·95 ± 1·54 a	3·77 ± 1·15 b	2·31 ± 0·64 a
cyclo 19:0	0·72 ± 0·43 a	0·03 ± 0·05 b	0·05 ± 0·08 b
Class G total	0·66 ± 0·35 a	1·01 ± 0·76 a	2·61 ± 1·45 b
iso-15:1‡	0·05 ± 0·05 a	0·34 ± 0·64 a	2·05 ± 1·49 b
Total 20-carbons	1·84 ± 0·74 a	0·53 ± 0·48 b	0·29 ± 0·16 b
Ratio class C/A + B	1·15 ± 0·13 a	1·80 ± 0·20 b	2·57 ± 0·40 c
C/A‡	1·17 ± 0·11 a	1·84 ± 0·22 b	2·66 ± 0·40 c

* Data from Caudales & Wells (1992).

† Percentage of total ± standard deviation. Averages in each row not followed by the same letter are statistically different ($P \leq 0.05$).

‡ Parameter of taxonomic value in distinguishing free-living *Anabaena* and *Nostoc* (Caudales & Wells, 1992).

14:0, 16:0 and 18:0 carbon chains; the unsaturated straight-chained 12:1, 14:1 *cis*-7, 16:1 *cis* 7, 16:1 *cis*-9, 16:1 *cis*-11, 16:3 *cis*-6, 18:2 *cis*-9, 18:3 *cis*-9, 18:1 *cis* and *trans*-9, and 20:4 *cis*-5; and the branch-chained *iso*-16:0 (Table 2). Also included was an unsaturated 16-carbon (ECL 15·5) with unsaturation sites undetermined. The most abundant component in the group was the 16:0, with a mean concentration of 38·10% of the total, and ranging between 35·17 and 42·16%.

There were 38 minor fatty acid components detected and identified, comprising 10·3% of the total, and 4 trace-level components. These included hydroxy-substituted acids (1·10%), branch-chains in addition to the *iso*-16:0 (1·96% of a total of 2·96%), and cyclopropane acids (0·89%) (Table 2).

Comparing the fatty acid composition of *Azolla* cyanobionts with those previously published for the free-living cyanobacteria *Anabaena* and *Nostoc*, cyanobionts differed from one or both of the cyanobacteria in the composition of 12 specific fatty acids, including the 4 previously identified as important in the differentiation of *Anabaena* and *Nostoc* (Caudales & Wells, 1992). Values for 16:0, total class A, and the ratio of classes C/A in *Azolla* cyanobionts were significantly different from those in *Anabaena* and *Nostoc* (Table 3). Percentages for 16:1 *cis*-9 and *iso*-15:1 were similar to those reported for *Anabaena*, but not for *Nostoc*; and the percentage of 14:1 *cis*-7 was similar to *Nostoc*, but not to *Anabaena*. Percentages of the 14:0, 16:1 *trans*-3, 20:4 *cis*-5, and cyclo-19:0 were also different in the cyanobiont from those in both *Anabaena* and *Nostoc*, as well as percentages for classes C and G (Table 3).

One fatty acid, *iso*-15:1, was either absent or present as a minor component in *Azolla* cyanobionts, while in *Nostoc* it constituted a major component (Caudales & Wells, 1992). Two fatty acids, 16:1 *trans*-3 and *iso* 3-OH 13:0, were present in cyanobionts but had not been previously reported in *Anabaena* or *Nostoc*. The 16:1 *trans*-9 was also either absent or present in only trace amounts in the cyanobionts.

Poly-unsaturated acids in the *Azolla* cyanobionts averaged 30·67% of the total, compared to 33·02% and 32·90% in *Anabaena* and *Nostoc*, respectively (data not shown). The most abundant were the 18:3 *cis*-9 and the 18:2 *cis*-9. The composition of total mono-unsaturated acids in cyanobionts was 17·30%, compared to 24·74% and 32·23% for *Anabaena* and *Nostoc*. The mean percentage of combined 16-carbon fatty acids was 50·95%.

Control preparations of cellular debris from *Azolla* and of bacterial contaminants from *Azolla* cavities yielded no fatty acid peaks in their respective chromatograms. Variations (standard deviations) in fatty acid percentages of the one cyanobiont repeatedly analysed (*A. rubra*) were within the ranges for the group in general.

Discussion

Oxygenic photosynthetic cyanobacteria have the same gross membrane organization as other Gram-negative prokaryotic eubacteria except for their thylakoid membrane sacs which house the electron transport chain, chlorophyll *a* and reaction centers (Drews & Weckesser, 1982; Golecki & Drews, 1982). Fatty acid composition is also different in cyanobacteria, possibly due in part to these photosynthetic membrane characteristics. In eubacteria, branch-chain fatty acids are major constituents, and straight chains may constitute up to 10% of total fatty acids (Kaneda, 1991). In the cyanobacteria, the 16- and 18-carbon straight chains were the major fatty acids, similar to those in plants (Christie, 1987), and the branch-chains were minor components. Another difference is the high percentage of polyunsaturated acids in cyanobacteria compared to their absence in most eubacteria (Johns & Perry, 1977; De Long & Yayanos, 1986). These fundamental differences in composition may have a direct bearing on the balance and fluidity of membrane lipids.

Cyanobionts are enclosed in dorsal cavities of *Azolla* fronds where the environmental medium and light

quality differs from that surrounding free-living cyanobacteria. Fatty acid composition of the cyanobionts may be significantly affected by these factors, possibly accounting for their differences with free-living cyanobacteria. Strong illumination, for example, during growth of *Anabaena variabilis* stimulated desaturation of 18:1 and 18:2 fatty acids (Sato & Murata, 1981). Differences in morphology may also influence fatty acid composition. Up to 30% of the cells of cyanobionts may be heterocysts (Hill, 1975) compared to 5–10% in free-living cyanobacteria (Fogg *et al.*, 1973). Fatty acid composition of cyanobionts may also be influenced by host species (study in progress).

Plant debris or other bacteria may also have contaminated the preparations of *Azolla* cyanobacteria. Although low levels of such contaminants (as determined by microscopy) remained after the purification procedure, their biomass was demonstrated to be too small to contribute to fatty acid profiles of the cyanobacteria. Only trace levels of fatty acids were obtained from plant or bacterial preparations, 2 to 3 orders of magnitude more concentrated than levels generally found in cyanobacterial preparations.

It is possible that the cyanobacteria isolated from *Azolla* are a mixed population (Gebhardt & Nierzwicki-Bauer, 1991), including epiphytes, rather than pure cultures. There is evidence that restriction fragment analysis of cultivable cyanobionts does not correspond to that of freshly isolated cells from *Azolla* cavities (Meeks *et al.*, 1988). However, since microscopic observations of our samples indicated morphological uniformity, and since the issue is unresolved, we assumed the cultures were essentially homogenous.

A comparison of fatty acid profiles of *Azolla* cyanobionts with those previously published for the free-living cyanobacteria *Anabaena* and *Nostoc*, indicated there were at least 19 factors – individual fatty acids, class totals, and ratios – which were statistically different (Table 3). Nine of the 19 factors were unique to the *Azolla* cyanobionts; i.e. different from both *Anabaena* and *Nostoc*. Five were different from only *Anabaena*, and five from only *Nostoc*. A numerical tally of differentiating factors, therefore, implies that cyanobionts were as different from *Anabaena* as from *Nostoc*.

Among the differentiating factors, the largest components were class A and C totals, accounting for over 90% of total fatty acids in *Azolla* cyanobionts. The composition of class A in the cyanobionts was different from both *Anabaena* and *Nostoc*, and class C was different from that of *Nostoc* only. Thus, an analysis of differentiating factors of quantitative importance indicates a greater resemblance to *Anabaena* than to *Nostoc*.

A third interpretion of the data, centering on the existence of 9 factors unique to the *Azolla* cyanobionts, suggests that fatty acid composition of the cyanobionts is sufficiently different from both *Anabaena* and *Nostoc* to hypothesize their constituting a distinct taxonomic grouping. This hypothesis would be at variance with conclusions based solely on morphological evidence (Caudales *et al.*, 1990), and on two RFLP studies (Meeks *et al.*, 1988; Plazinski *et al.*, 1990) that cyanobionts are more closely related to *Nostoc* than to *Anabaena*.

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